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MyD88: An Adapter That Recruits IRAK to the IL-1 Receptor Complex

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Summary

IL-1 is a proinflammatory cytokine that signals through a receptor complex of two different transmembrane chains to generate multiple cellular responses, including activation of the transcription factor NF-κB. Here we show that MyD88, a previously described protein of unknown function, is recruited to the IL-1 receptor complex following IL-1 stimulation. MyD88 binds to both IRAK (IL-1 receptor–associated kinase) and the heterocomplex (the signaling complex) of the two receptor chains and thereby mediates the association of IRAK with the receptor. Ectopic expression of MyD88 or its death domain–containing N-terminus activates NF-κB. The C-terminus of MyD88 interacts with the IL-1 receptor and blocks NF-κB activation induced by IL-1, but not by TNF. Thus, MyD88 plays the same role in IL-1 signaling as TRADD and Tube do in TNF and Toll pathways, respectively: it couples a serine/threonine protein kinase to the receptor complex.

Introduction

Interleukin-1 (IL-1) participates in defense response to environmental challenges by generating fever, activating lymphocytes, and promoting infusion of leukocytes into the sites of injury or infection (reviewed by Dinarello, 1996). IL-1 exerts most of its activities by activating the transcription of genes encoding chemokines, cytokines, acute-phase proteins, cell adhesion molecules, and enzymes involved in the production of small proinflammatory substances (reviewed by Dinarello, 1996; Barnes and Karin, 1997). One important intracellular mediator of the IL-1 response is nuclear transcription factor κB (NF-κB) (reviewed by Baeuerle and Baltimore, 1996; Baldwin, 1996; Dinarello, 1996; Barnes and Karin, 1997), which is rendered inactive in the cytoplasm by the inhibitory proteins called IκB in unstimulated cells. Upon exposure of cells to cytokines and other extracellular stimuli, the IκB proteins are phosphorylated on specific serine residues and rapidly degraded, leading to nuclear localization and activation of NF-κB (reviewed by Baeuerle and Henkel, 1994; Siebenlist et al., 1994; Thanos and Maniatis, 1995; Verma et al., 1995).

Although the biochemical process of NF-κB activation has been well characterized, the receptor proximal signaling mechanisms for the various cytokines remain undefined. The elucidation of the IL-1 signaling pathway just began within the past few years. The first signaling event for IL-1 is a ligand-induced complex formation of the type I receptor (IL-1RI) and the receptor accessory protein (IL-1RAcP) (Greenfeder et al., 1995; Huang et al., 1997; Korherr et al., 1997; Wesche et al., 1997). The serine/threonine IL-1 receptor–associated kinase (IRAK) is then recruited to the receptor complex, where it becomes highly phosphorylated (Cao et al., 1996a; Yamin and Miller 1997). IRAK then leaves the receptor complex and interacts with TRAF6, which is required for IL-1–induced NF-κB activation (Cao et al., 1996b). Two other members of the TRAF (TNF-receptor associated factor) family, TRAF2 and TRAF5, have been implicated in NF-κB activation signaled by the tumor necrosis factor (TNF) receptor superfamily (Rothe et al., 1995; Hsu et al., 1996a, 1997; Ishida et al., 1996; Lee et al., 1996; Nakano et al., 1996; Aizawa et al., 1997; Duckett et al., 1997). These findings indicate that signaling pathways for different NF-κB-activating cytokines may converge at the TRAF molecules.

Recently, a kinase complex consisting of the NF-κB–inducing kinase (NIK) and two IκB kinases (IKKα and IKKβ) have been implicated in signal-induced phosphorylation of the IκB proteins (Di Donato et al., 1997; Malinin et al., 1997; Mercurio et al., 1997; Regnier et al., 1997; Woronicz et al., 1997; Zandi et al., 1997). NIK interacts with the TRAF proteins (Malinin et al., 1997; Song et al., 1997), and IKKs phosphorylate IκB proteins on specific serine residues. Thus, this kinase cascade constitutes a section of the signaling pathways distal to the TRAF molecules.

IL-1RI and IL-1RAcP share an overall 25% sequence identity. Both belong to a conserved family of receptor-like molecules, including IL-1Rrp, IL-1Rrp2, T1/ST2, rsc786/TIL, and human Toll in mammals (Yanagisawa et al., 1993; Lovenberg et al., 1996; Mitcham et al., 1996; Parnet et al., 1996; Medzhitov et al., 1997); RPP5 in plants (Parker et al., 1997); and 18 Wheeler and Toll in Drosophila melanogaster (Hashimoto et al., 1988; Eldon et al., 1994). Among these molecules, the Toll signaling pathway was, for a long time, the best characterized. The Toll pathway is required for activation of the antifungal peptide drosomycin in response to microorganism challenges (Lemaitre et al., 1996). This pathway also controls dorsal–ventral polarization during early embryonic development (reviewed by Wasserman, 1993; Morisato and Anderson, 1995; Belvin and Anderson, 1996; Norris and Manley, 1996). Analogous to IL-1 signaling, activation of the cell surface receptor Toll by the extracellular ligand Spaetzle leads to the phosphorylation and degradation of the IκB-like molecule Cactus, releasing the NF-κB–like transcription factor Dorsal to enter the nucleus, where it regulates gene expression. Dorsal activation signaled by Toll requires two intermediate signal transducers, Tube and Pelle (Letsou et al., 1993; Shelton and Wasserman, 1993). Pelle is a serine/threonine kinase that is related by sequence to IRAK, whereas

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Tube is an adapter molecule that tethers Pelle to the plasma membrane (Grosshans et al., 1994; Galindo et al., 1995). Tube and Pelle interact through their N-terminal death domains, a protein–protein interaction domain found in many signaling molecules, including IRAK (Feinstein et al., 1995; Galindo et al., 1995; Cao et al., 1996a). Because of the similarities found at multiple levels in the signaling pathways initiated by Toll and IL-1R, they probably evolved from the same ancestral signaling mechanism and probably use conserved intermediate components. However, to date, the mammalian homolog of Tube has not been identified.

Here we report the identification of MyD88, a previously described myeloid differentiation marker (Lord et al., 1990), as a signaling molecule for IL-1. We show that MyD88 is recruited to the IL-1 receptor complex by IL-1 and functions as an adapter to recruit IRAK. Thus, MyD88 represents a functional mammalian analog of Drosophila Tube. Moreover, the role of MyD88 in IL-1 signaling is similar to that of the TNF signaling molecule TRADD (type 1 TNF receptor–associated death domain protein), which recruits the kinase RIP (receptor-interacting protein) to the type I TNF receptor complex (Hsu et al., 1995). These findings reveal that two cytokines with similar biological functions, IL-1 and TNF, use analogous signaling mechanisms to generate cellular responses.

Results

Identification of MyD88 in the IL-1 Receptor Complex

To identify potential novel signaling proteins in the IL-1 receptor complex, we generated a human embryonic kidney epithelial 293 cell line (293IL-1RI/AcP) stably expressing Myc-epitope–tagged IL-1RI and Flag-epitope–tagged IL-1RAcP. These cells have higher basal NF-κB activity but still respond to IL-1 with a further increase in NF-κB activation (data not shown). The 293IL-1RI/AcP cells were metabolically labeled with [^{35}S]methionine and cysteine and treated for 3 min with IL-1 or left untreated before harvesting. Aliquots of cell extracts were immunoprecipitated with polyclonal antibodies to IRAK, TRAF6, or the extracellular domain of IL-1RI, or with anti-Flag monoclonal antibodies (MAb). Autoradiography of the immunoprecipitates separated on a sodium dodecyl sulfate (SDS) gel revealed a 35 kDa protein (p35) coprecipitating with IL-1RI, IL-1RAcP, and IRAK in an IL-1–dependent manner (Figure 1A). p35 was not detected in the immunoprecipitates of normal rabbit serum or TRAF6 antiserum. Because IL-1RI, IL-1RAcP, and IRAK form a biochemically detectable complex upon IL-1 treatment (Huang et al., 1997), whereas TRAF6 is not stably associated with this complex (Cao et al., 1996b), p35 represented a good candidate for a receptor-associated signaling molecule.

To purify p35, protein extracts from untreated or IL-1–treated 293IL-1RI/AcP cells were precipitated with anti-Flag antibody immobilized on sepharose (M2 beads). After extensive washing, the precipitated IL-1RAcP complex was eluted with Flag peptide, concentrated, and resolved by SDS polyacrylamide gel electrophoresis

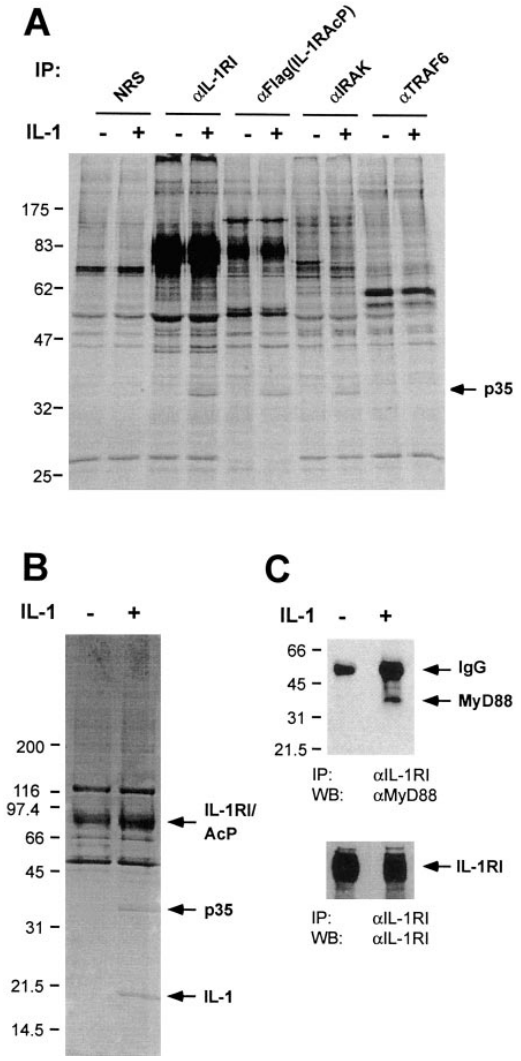

Figure 1. Identification of MyD88 in the IL-1 Receptor Complex

(A) Coprecipitation of a 35 kDa protein with the IL-1 receptor complex. 293IL-1RI/AcP cells were metabolically labeled with ^{35}S-radiolabeled amino acids, stimulated with IL-1β (3 min, 200 ng/ml), or left untreated. Cell lysates were subjected to immunoprecipitation with normal rabbit serum (NRS), polyclonal antisera raised against huIL-1RI, IRAK, or huTRAF6, and MAb specific for the Flag epitope that was tagged on IL-1RAcP. Immunoprecipitates were separated by SDS-PAGE and labeled proteins were visualized by autoradiography. Arrow, the 35 kDa protein coprecipitating with IL-1RI, IL-1RAcP, and IRAK.

(B) Purification of p35. Equal amounts of untreated or IL-1–induced 293IL-1RI/AcP cells were lysed and the extracts immunoprecipitated with the M2 beads. Immunocomplexes were eluted with the Flag peptide, separated by SDS-PAGE, and stained with Coomassie brilliant blue. The positions of IL-1RI, IL-1RAcP, p35, and IL-1 are indicated.

(C) IL-1–induced association of MyD88 with the IL-1 receptor complex. IL-1 receptor complexes from lysates of IL-1–treated (200 ng/ml IL-1β, 5 min) and untreated 293IL-1RI cells were immunoprecipitated with anti IL-1RI antiserum and immunoblotted with antiserum to MyD88 (top). Western analysis of the same blot with antiserum to IL-1RI indicates that similar amounts of IL-1RI were immunoprecipitated from untreated and IL-1–treated cells (bottom).

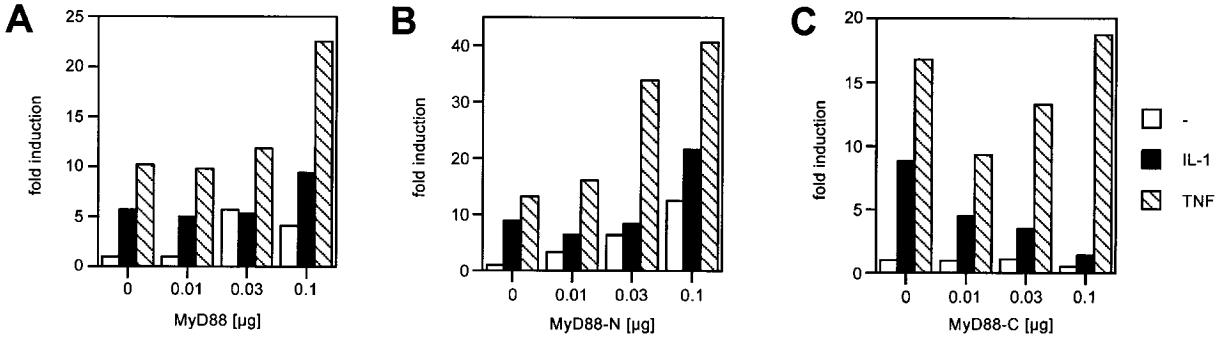

Figure 2. NF-κB Activation by MyD88

HeLa cells (1×10^5) were transfected with 0.5 μg of pELAM-luc reporter gene plasmid, 1 μg of pRSV-β-gal, and different amounts of expression plasmids for either full-length MyD88 (A), MyD88-N (amino acids 1–151) (B), or MyD88-C (amino acids 152–296) (C). Forty hours after transfection cells were left untreated or were stimulated for 8 hr with IL-1β (20 ng/ml) or TNF (100 ng/ml). Luciferase activities were determined and normalized on the basis of β-galactosidase activity. Data shown are from one of five independent experiments with similar results.

(SDS-PAGE). Coomassie blue staining revealed three bands showing increased intensity upon IL-1 treatment: p35, a diffuse band of 70–80 kDa corresponding to IL-1RI and IL-1RAcP, which are similar in size after glycosylation, and a 17 kDa protein that is identical in size to IL-1 (Figure 1B).

The p35 protein was digested with lysine C and the resulting peptides were separated by high-performance liquid chromatography. A search of a protein sequence database by peptide mass fingerprinting (Henzel et al., 1993) matched p35 with a known protein, designated MyD88, with a calculated mass of 33 kDa. This identity was verified by sequence analysis of a peptide with a mass of 2832.1 (LCVXDRDVLP) and post–source decay analysis (Kaufmann et al., 1994) of a peptide with a mass of 1255.8. To confirm the peptide analysis and sequencing results, the receptor complexes were immunoprecipitated with antiserum to IL-1RI from untreated or IL-1–treated 293IL-1RI cells and immunoblotted with antiserum to MyD88. A 35 kDa band reactive to the specific antiserum was detected in the receptor complex derived from IL-1 treated but not untreated cells (Figure 1C), indicating that p35 is indeed MyD88.

Identification of MyD88 as an IL-1 Signaling Molecule

MyD88 was first identified as a myeloid differentiation primary response gene (Lord et al., 1990). Its expression was subsequently detected in a variety of nonmyeloid tissues, indicating that the protein might have a broader biological function (Hardiman et al., 1996; Bonnert et al., 1997). By sequence analysis, the protein contains two functional domains, an N-terminal death domain and a C-terminal domain homologous to the cytoplasmic tails of the IL-1 receptor family (Hultmark, 1994; Hardiman et al., 1996). Although it has been reported that ectopic expression of MyD88 activates an NF-κB–dependent IL-8 gene promoter in cos7 cells (Bonnert et al., 1997), its biological role remains unclear. The detection of MyD88 in the activated IL-1 receptor complex provided biochemical evidence that MyD88 might participate in IL-1 signal transduction. We then used the luciferase reporter assay to investigate the functional role of MyD88 in IL-1–induced NF-κB activation.

Transient transfection of 293 cells with small amounts of mammalian expression plasmids for MyD88 activated a cotransfected NF-κB–dependent luciferase gene in a dose-dependent manner (Figure 2A). Treatment of the transfected cells with IL-1 or TNF further enhanced the luciferase activity. We next sought to determine which portion of MyD88 protein is required for the observed activity. Expression of the N-terminal 151 amino acids of MyD88 (MyD88-N), including the death domain, elicited NF-κB activation similar to that by the full-length protein (Figure 2B). However, the C-terminal half (amino acids 152–296, MyD88-C), including the IL-1 receptor homology domain, not only failed to activate NF-κB but also specifically blocked the NF-κB activation by IL-1, yet had no effect on NF-κB activation by TNF. This specific dominant negative effect of MyD88 C-terminus on IL-1 signaling further indicates that MyD88 is a component in the IL-1 pathway.

Association of MyD88 with the IL-1 Receptor Complex

To investigate the role of MyD88 in IL-1 signaling, we determined the kinetics of IL-1–induced MyD88 recruitment to the IL-1 receptor complex. The receptor complexes in 293IL-1RI/AcP cells stimulated with IL-1 for various lengths of time were immunoprecipitated with anti-IL-1RI antiserum and immunoblotted with antiserum to MyD88. MyD88 was detected 30 s after the IL-1 treatment, and the amounts of the protein increased slightly but steadily over the next 10 min (Figure 3A, top). This result indicates that IL-1–induced association of MyD88 with the receptor complex is rapid and relatively stable. On the other hand, immunoblotting of the same membrane with antiserum to IRAK indicates that the interaction of IRAK with the receptor complex is rapid but transient. In this cell line, the amounts of receptor-associated IRAK peaked 30 s after IL-1 induction, followed by a steep decline (Figure 3A, bottom). This result agrees with our earlier speculation that interaction of IRAK with the downstream TRAF6 occurs in the cytoplasm off the receptor complex (Cao et al., 1996b). A mobility shift of IRAK that reflects autophosphorylation was evident 30 s after IL-1 treatment (unphosphorylated IRAK runs as an 80 kDa band in an SDS gel [Cao et al., 1996a]). A further shift of mobility was observed at later time points.

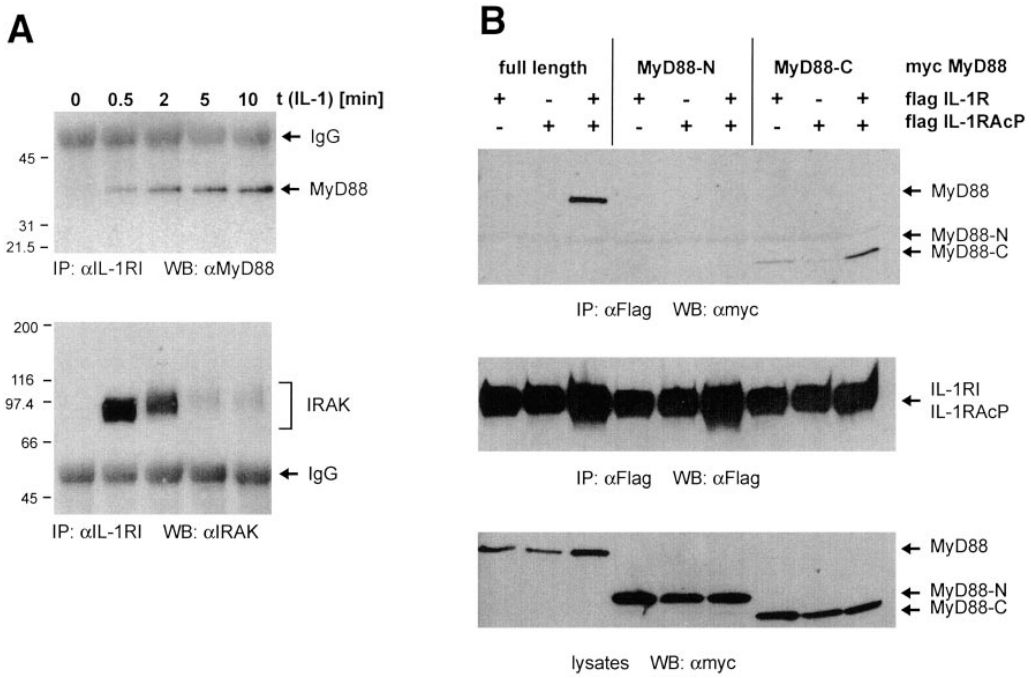

Figure 3. Interaction of MyD88 with the IL-1 Receptor Complex

(A) Kinetics of IL-1–induced MyD88 and IRAK association with the IL-1 receptor complex. 293IL-1RI/AcP cells (6×10^7) were stimulated with 200 ng/ml IL-1β for the indicated times. IL-1 receptor complexes from cell lysates were immunoprecipitated with an anti-IL-1RI antiserum and immunoblotted with antiserum to MyD88 (top) and IRAK (bottom).

(B) Interaction of MyD88 with heterocomplexes of the receptor chains. 293 cells (3×10^6) in a 10 cm dish were transfected with the indicated combinations of expression plasmids for Flag-tagged IL-1RI; IL-1RAcP; and Myc-tagged MyD88, MyD88-N (amino acids 1–151), or MyD88-C (amino acids 152–296) (1 μg of each expression construct per transfection). After 24 hr, cell lysates were prepared and immunoprecipitations were performed with anti-Flag MAb. Coprecipitating Myc-tagged MyD88 was detected with anti-Myc antiserum (top). Immunoblotting the same membrane with anti-Flag MAb indicates that similar amounts of the receptor chains were immunoprecipitated (middle). Lysates of the transfected cells were immunoblotted with Anti-Myc antiserum to monitor the expression of MyD88 (bottom).

IL-1 signal transduction requires both IL-1RI and IL-1RAcP, which form a heterocomplex upon IL-1 treatment (Greenfeder et al., 1995; Huang et al., 1997). To understand the mechanism by which MyD88 is recruited to the receptor complex, we examined the binding affinity of MyD88 to IL-1RI, IL-1RAcP, and the heterocomplex of these two receptor chains. 293 cells were transiently transfected with expression plasmids for Myc epitope–tagged MyD88 with expression plasmids for N-terminal (extracellular) Flag-tagged IL-1RI and IL-1RAcP. The receptor complexes were immunoprecipitated with anti-Flag antibody and immunoblotted with anti-Myc antibody. MyD88 coprecipitated with neither IL-1RI nor IL-1RAcP when they were individually expressed but was readily detectable in anti-Flag immunoprecipitates from cells that expressed both receptor chains (Figure 3B). When IL-1RI and IL-1RAcP are transiently coexpressed in 293 cells, they form a functional complex able to activate NF-κB (Huang et al., 1997). The observed high affinity of MyD88 to the heterocomplex of receptors provides a mechanism for IL-1–dependent recruitment of MyD88.

To determine which domain of MyD88 is responsible for its receptor association, we tested whether MyD88-N or MyD88-C is able to bind to the receptor complex, using the same experimental procedure. The N-terminal half of MyD88 did not coprecipitate with the receptors even when the two chains were coexpressed. The C-terminal half, however, bound weakly to IL-1RI alone but bound with high affinity to the IL-1RI and IL-1RAcP heterocomplex (Figure 3B), indicating that the receptor homology region of MyD88 is responsible for its interaction with the IL-1 receptor complex.

Inability of Inactive Receptor Mutants to Bind to MyD88

The intracellular domains of both IL-1RI and IL-1RAcP are required for IL-1 signal transduction (Curtis et al., 1989; Heguy et al., 1992; Kuno et al., 1993; Leung et al., 1994; Huang et al., 1997). Deletion of 45 amino acids from the C-terminus of the IL-1RI abolishes the ability of the receptor to signal (Croston et al., 1995). Similarly, removal of 37 amino acids from the C-terminus of IL-1RAcP creates a loss-of-function mutant (data not shown). Overexpression of either IL-1RIΔ45 or IL-1RAcPΔ37 also inhibits IL-1–induced NF-κB activation in a reporter assay (Figure 4A). This result indicates that the mutants are still capable of binding ligand and forming a receptor complex.

To investigate whether these receptor mutants fail to signal because they can no longer recruit MyD88, we tested whether receptor complexes containing IL-1RIΔ45 and IL-1RAcPΔ37 can bind to MyD88. 293 cells were transfected with expression plasmids for Myc-tagged

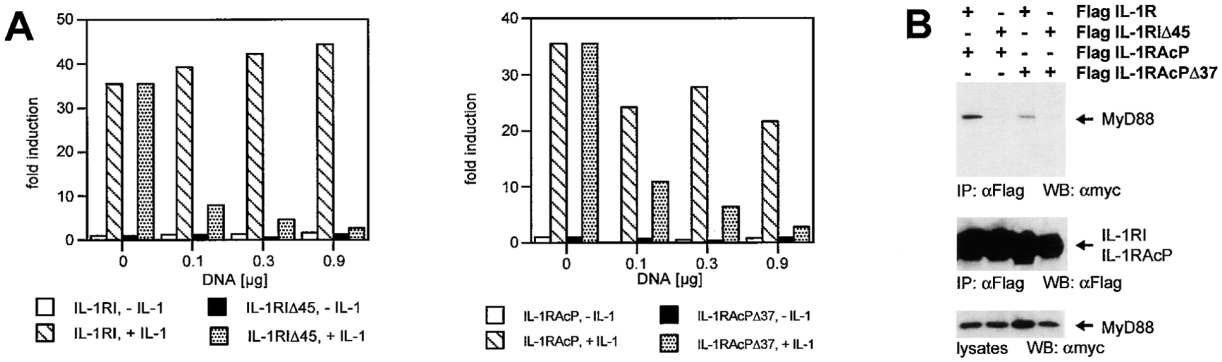

Figure 4. Interaction of MyD88 with the Functional Receptor Complex

(A) Inhibition of IL-1–induced NF-κB activation by IL-1RIΔ45 (left) and IL-1RAcPΔ37 (right). HeLa cells were transfected with 0.5 μg of pELAM-luc reporter gene plasmid; 1 μg of pRSV-β-gal; and varied amounts of expressions plasmids for either IL-1RI, IL-1RIΔ45, IL-1RAcP, or IL-1RAcPΔ37 as indicated. Forty hours after transfection cells were either left untreated or were stimulated for 8 hr with IL-1β (20 ng/ml). Luciferase activities were determined and normalized on the basis of β-galactosidase activity. Similar results were obtained from four independent experiments.

(B) Inability of MyD88 to coprecipitate with receptor mutants. 293 cells (3×10^6) were transfected with 1 μg of expression plasmids for Myc-tagged MyD88 in combination with Flag-tagged wild-type and mutant IL-1RI and IL-1RAcP as indicated. After 24 hr, cell lysates were prepared and immunoprecipitated with anti-Flag MAb. Coprecipitating Myc epitope–tagged MyD88 was detected using Myc antiserum (top). Western analysis of the same blot with MAb to the Flag epitope indicates that similar amounts of the receptor chains were immunoprecipitated (middle). Immunoblot analysis of total cell extracts with anti-Myc antiserum shows that equal amounts of MyD88 were expressed (bottom).

MyD88 with various combinations of the Flag-tagged wild-type receptor chains, IL-1RIΔ45, and IL-1RAcPΔ37. Compared with the amounts of MyD88 coprecipited with the wild-type receptors, only very weak binding of MyD88 was detected in receptor complexes containing one or both loss-of-function mutants, establishing a correlation of receptor function and their ability to interact with MyD88 (Figure 4B).

Interaction of the IL-1R Complex with IRAK Mediated by MyD88

Because both MyD88 and IRAK coprecipitate with the receptor complex in an IL-1–dependent manner (Figures 1C and 3A), we investigated whether MyD88 might play the role of an adapter for IRAK. Flag-tagged full-length or truncational mutants of MyD88 were coexpressed in 293 cells with either wild-type or a kinase-defective mutant of IRAK (IRAK K239S). Immunoblotting analysis of the anti-Flag immunoprecipitates showed that the full-length protein and the N- and C-terminal portions of MyD88 each can bind to the kinase defective form of IRAK.

Of note, MyD88 did not coprecipitate the kinase-active form of IRAK, which is spontaneously autophosphorylated upon overexpression, as evidenced by its retarded mobility in an SDS gel (Figure 5A, bottom). Because endogenous IRAK remains underphosphorylated until it is recruited into the receptor complex (Cao et al., 1996a), the high affinity of MyD88 for underphosphorylated IRAK is consistent with its role as an adapter for recruiting IRAK. In addition, the inability of MyD88 to bind to phosphorylated IRAK may explain how IRAK leaves the receptor complex after its phosphorylation.

If MyD88 is an adapter for IRAK, then overexpression of MyD88 should reduce the amounts of endogenous IRAK coprecipitating with the IL-1 receptor complex upon IL-1 stimulation, given the limiting amounts of IRAK and receptor molecules in cells. To test this idea, the various MyD88 expression vectors were transfected into 293IL-1RI cells. After 36 hr, the cells were treated for 5 min with IL-1 or left untreated and lysed. The extracts were immunoprecipitated with anti-IL-1RI antiserum and the precipitates immunoblotted to detect IRAK. Compared with the amounts of IRAK in the receptor complex in cells transfected with an empty vector, reduced amounts of IRAK were detected in the receptor complex from cells expressing the full-length protein, the N-terminal half, and the C-terminal half of MyD88 (Figure 5B). Considering that transfection efficiency with 293 cells is typically 50%–75%, the actual blocking effect of MyD88 on IRAK binding to the receptors is probably far greater than represented here.

To examine directly whether MyD88 can mediate the association of IRAK and IL-1R, we transiently coexpressed the two Flag-tagged receptor chains with IRAK or IRAK K239S in the presence or absence of MyD88. Immunoprecipitation with anti-Flag antibody coprecipitated IRAK K239S only in the presence of MyD88 (Figure 5C). The phosphorylated kinase active IRAK that did not bind to MyD88 (Figure 5A) also did not coprecipitate with the receptor chains even in presence of MyD88. These results indicate that MyD88 is an intermediate signaling molecule linking IRAK and the receptor complex.

The Hierarchy of MyD88, IRAK, and TRAF6 in IL-1 Signaling Pathway

The biochemical evidence presented above suggests that MyD88 couples IRAK to the receptor and therefore functions upstream of IRAK. We used the reporter gene assay to examine this hierarchy. Overexpression of the N-terminal 215 amino acids of IRAK (IRAK-N) and the C-terminus of TRAF6 (amino acids 289–522, ΔTRAF6) inhibited IL-1–induced NF-κB–dependent luciferase activity in 293IL-1RI cells (Figure 6A) (Cao et al., 1996b).

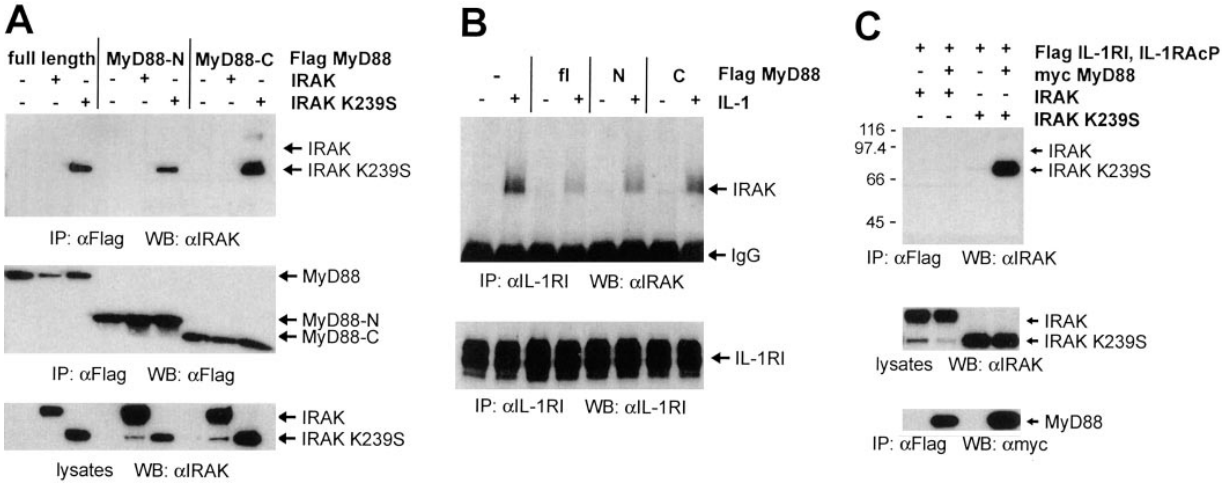

Figure 5. IRAK/Receptor Interaction Mediated by MyD88

(A) Coprecipitation of MyD88 and IRAK. 293 cells (3×10^6) were transfected with 2 μg of expression plasmid for Flag-tagged MyD88, MyD88-N (amino acids 1–151), or MyD88-C (amino acids 152-296) alone or together with 3 μg of expression plasmid for wild-type IRAK or IRAK K239S. After 24 hr, cell lysates were prepared and immunoprecipitated with anti-Flag MAb. Coprecipitating IRAK was detected using an IRAK antiserum (top). Western analysis of the same blot with MAb to the Flag epitope shows that similar amounts of MyD88 were immunoprecipitated (middle). The lysates of the transfected cells were immunoblotted with IRAK antiserum to monitor the expression of IRAK (bottom).

(B) Influence of MyD88 overexpression on IL-1–induced recruitment of endogenous IRAK to the IL-1 receptor complex. 293IL-1RI cells (3×10^6) were transfected with 2 μg of empty vector or expression plasmid for Flag-tagged MyD88, MyD88-N (amino acids 1–151) or MyD88-C (amino acids 152–296) as indicated. IL-1 receptor complexes from lysates of IL-1 treated (200 ng/ml IL-1β, 5 min) and untreated cells were immunoprecipitated with an anti–IL-1RI antiserum and immunoblotted with IRAK antiserum (top). Western analysis of the same blot with antiserum to IL-1RI indicates that similar amounts of IL-1RI were immunoprecipitated (bottom).

(C) IRAK/receptor interaction through MyD88. 293 cells were transiently transfected with expression plasmids for Flag-tagged IL-1RI and IL-1RAcP in various combinations with expression plasmids for IRAK, IRAK K239S, and Myc-tagged MyD88 as indicated. The receptor complexes were precipitated with anti-Flag M2 beads, separated on an SDS gel, and immunoblotted with antiserum to IRAK (top). The expression of IRAK (middle) and MyD88 (bottom) was verified by immunoblotting analysis of the cell lysates.

When IRAK-N or ΔTRAF6 was coexpressed with MyD88, NF-κB activation by MyD88 was essentially abolished, suggesting that MyD88 signals through IRAK and TRAF6 (Figure 6B). To examine this order of signaling events further, we tested whether the dominant negative mutant MyD88-C or ΔTRAF6 could block IRAK-induced NF-κB activation. Transient expression of IRAK induced a mild, 5- to 6-fold NF-κB activation. Coexpression of

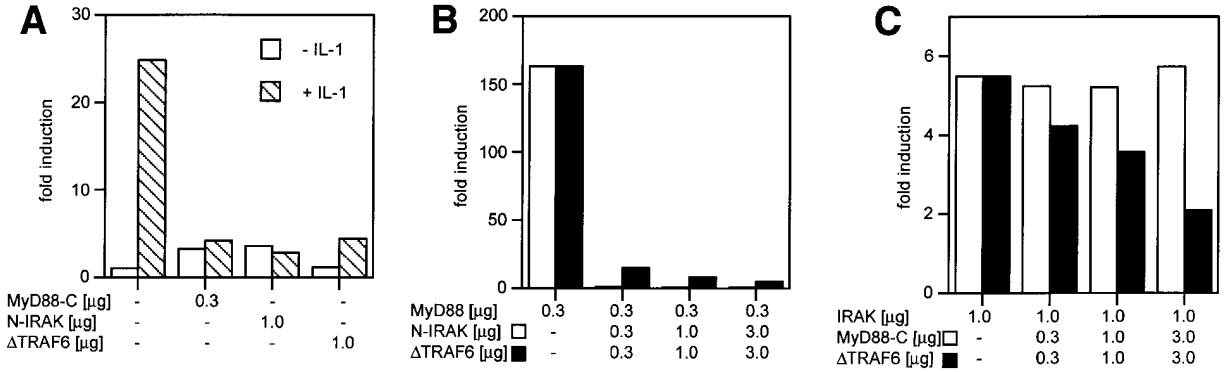

Figure 6. Hierarchy of MyD88, IRAK, and TRAF6 in IL-1 Signaling

(A) Dominant negative effect of MyD88-C, N-IRAK, and ΔTRAF6 on IL-1–induced NF-κB activation. 293 cells (1×10^5) were transfected with 0.5 μg of pELAM-luc reporter gene plasmid, 1 μg of pRSV-β-gal, and different amounts of expression plasmids for MyD88-C, N-IRAK, or ΔTRAF6 as indicated. Twenty hours after transfection, cells were left untreated or were stimulated for 8 hr with IL-1β (20 ng/ml). Luciferase activities were determined and normalized on the basis of β-galactosidase activity.

(B) Abrogation of MyD88-induced activation of NF-κB by N-IRAK and ΔTRAF6. 293 cells were transfected with 0.5 μg of pELAM-luc reporter gene plasmid, 1 μg of pRSV-β-gal, 0.3 μg of a vector encoding MyD88, and varied amounts of expression plasmids for N-IRAK or ΔTRAF6 as indicated. Reporter assays were performed as described for (A).

(C) Effects of MyD88-C and ΔTRAF6 expression on IRAK-induced NF-κB activation. 293 cells were transfected with 1 μg of IRAK expression plasmid with the indicated amounts of MyD88-C and ΔTRAF6 expression vectors. Reporter assays were carried out as described for (A). Three independent experiments produced similar results.

ΔTRAF6 counteracted this IRAK effect in a dose-dependent manner. In contrast, MyD88-C, which dramatically blocked IL-1–induced NF-κB activation (Figures 2C and 6A), exerted no effect on IRAK activity, a result consistent with the notion that MyD88 functions upstream of IRAK.

Discussion

IL-1 is a potent NF-κB activator that signals through two related receptor chains, IL-1RI and IL-1RAcP. Both proteins contain large cytoplasmic domains (212 and 187 amino acids for IL-1RI and IL-1RAcP, respectively) that are required for signaling (Curtis et al., 1989; Heguy et al., 1992; Kuno et al., 1993; Leung et al., 1994; Huang et al., 1997). Neither receptor chain possesses enzymatic activity, so they must engage other intracellular molecules to transduce IL-1 signals. We have previously identified the serine/threonine kinase IRAK, which is recruited to the receptor complex upon IL-1 induction (Cao et al., 1996a). The recruitment of IRAK requires the intracellular domains of both receptor chains. Receptor molecules with mutations in their intracellular domains failed to recruit IRAK and to signal (Croston et al, 1995; Huang et al., 1997) (Figure 4). However, because these experiments were performed in mammalian cells, it was difficult to assess whether IRAK binds to the receptor chains directly or is recruited by an adapter molecule. Given our knowledge about the Toll pathway in Drosophila, which is analogous to the IL-1 pathway at the levels of the receptor (Toll, IL-1RI, and IL-1RAcP), intermediate kinase (Pelle and IRAK), and downstream targets (Cactus/Dorsal and IκB/NF-κB), it seemed likely that IRAK is recruited to the receptor complex through an adapter molecule like Tube.

By metabolic cell labeling, we detected a 35 kDa protein coprecipitating with IL-1RI, IL-1RAcP, and IRAK, three known components of the active IL-1 receptor complex. Peptide sequencing and immunobotting with specific antiserum indicated that p35 is MyD88 (Figure 1C), a known protein with unknown function. We present several lines of evidence supporting the role of MyD88 as an IL-1 signal transducer. First, it binds exclusively to the heterocomplexes of IL-1RI and IL-1R1AcP (Figure 3B), which form only after IL-1 treatment (Greenfeder et al., 1995; Huang et al., 1997). Second, it binds to the wild-type receptor complex but not to the functionally crippled complex in which either receptor chain has been mutated by a truncation (Figure 4), suggesting that the binding to MyD88 is critical for receptor function. Third, both MyD88 and IRAK are detectable in the receptor complex within seconds after IL-1 stimulation (Figure 3A). Fourth, MyD88 binds to underphosphorylated IRAK and mediates its association with the receptor complex (Figure 5). Finally, the C-terminal half of MyD88 behaves as a dominant negative mutant that inhibits NF-κB activation induced by IL-1, but not by TNF (Figure 2). Together, these data consistently indicate that MyD88 participates in IL-1 signaling by connecting the receptors with a downstream kinase, IRAK. Further support of this model comes from the results of luciferase reporter assays, which indicate that MyD88 functions upstream of IRAK and TRAF6 (Figure 6).

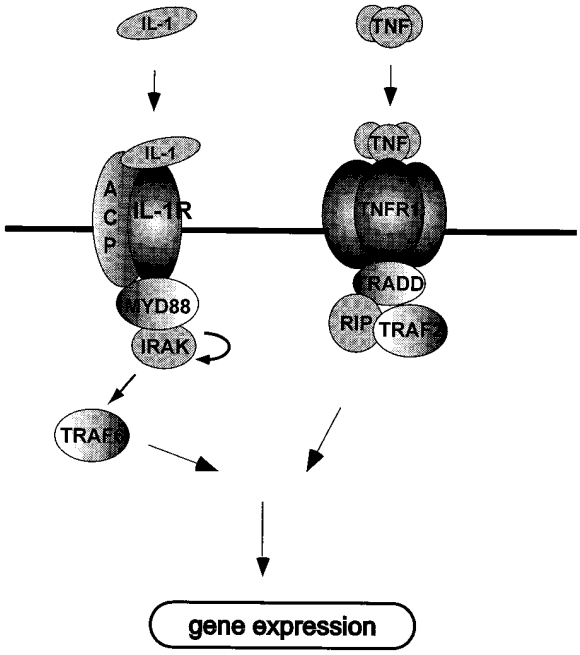

Figure 7. Receptor Proximal Signaling Pathway for IL-1 and TNF

The identification of MyD88 as an adapter molecule that recruits IRAK to the receptor complex extends the conservation found in the signaling pathways for IL-1 and TNF, two major NF-κB–activating cytokines (Figure 7). Whereas IL-1 signals through two different receptor chains, TNF signals by trimerizing the type 1 TNF receptor (TNFR1), which contains an intracellular death domain (Tartaglia and Goeddel, 1992). The TNFR1 complex then recruits the 34 kDa death domain–containing molecule TRADD through homophilic interactions of the death domain (Hsu et al., 1995). TRADD mediates two major TNF responses: NF-κB activation and apoptosis. To cause cell death, TRADD interacts with FADD to trigger the apoptotic protease cascades (Boldin et al., 1996; Grimm et al., 1996; Hsu et al., 1996a; Muzio et al., 1996). To activate NF-κB, TRADD interacts with serine/threonine kinase RIP and TRAF2, which ultimately feed into the NIK/IKK cascade to trigger the phosphorylation and degradation of the IκB proteins (Hsu et al., 1996a, 1996b). Because MyD88 is responsible for recruiting IRAK, which in turn interacts with TRAF6, it appears to be the functional equivalent of TRADD in NF-κB activation signaled by IL-1.

Of note, the binding of MyD88 to IL-1 receptors is also mediated by a homophilic interaction through a conserved region, the IL-1 receptor homology domain, which is located in the cytoplasmic tail of the receptor chains and the C-terminus of MyD88. This region in IL-1RI is critical for IL-1 signal transduction, since mutations of this region abolishes the receptor function (Croston et al., 1995). The N-terminal death domain of MyD88 mediates NF-κB activation upon overexpression and binds to IRAK, suggesting that MyD88 signals by engaging IRAK through its death domain. Overexpression of TRADD or the TRADD death domain that interacts with RIP also results in NF-κB activation, once again

revealing conserved properties shared by the counterparts of the IL-1 and TNF pathways. However, binding of IRAK apparently is not sufficient for NF-κB activation because the IRAK-binding MyD88 C-terminus is a dominant negative mutant that inhibits IL-1–induced NF-κB activation, suggesting that proper confirmation of the MyD88 and IRAK complex is important for signaling. Unlike TRADD, however, ectopic expression of MyD88 does not cause cell death in 293 and HeLa cells (data not shown). Similarly, IL-1 does not signal apoptosis in these cells.

The analogy between IL-1 and TNF signaling pathway outlined above and in Figure 7 implies that RIP and IRAK may play the same role in NF-κB activation signaled by TNF and IL-1, respectively. Although the two kinases show no similarity at the level of primary sequence, both are recruited to the receptors by adapter molecules, interact with TRAF proteins, and activate NF-κB when ectopically expressed in cells. Gene inactivation studies indicate that RIP is an essential molecule for TNF-induced NF-κB activation (Ting et al., 1996). However, the exact role of this molecule remains undefined. Elucidation of the mechanistic role of IRAK in IL-1 signaling may shed light on RIP function, and vice versa.

The number of identified IL-1 receptor–related molecules is rapidly increasing. To date, the biological functions of most of them have not been clearly defined. However, IL-1Rrp has recently been shown to be the receptor for an IL-1–related cytokine, IL-18, which synergizes with IL-12 to promote the production of interferon-γ by T cells (Okamura et al., 1995; Torigoe et al., 1997). Like IL-1 and TNF, IL-18 also activates NF-κB (Robinson et al., 1997; Torigoe et al., 1997). Given the homophilic nature of the MyD88/IL-1–receptor interaction, it is possible that MyD88 or related molecules may participate in the signaling of other IL-1 receptor family members. This is particularly true for IL-1Rrp because IL-18 stimulation of Th1 cells also activates IRAK (Robinson et al., 1997). In this light, elucidation of the IL-1 pathway provides a starting point to understand the signaling mechanisms for a large class of cytokine receptors.

Experimental Procedures

Biological Reagents and Cell Culture

Recombinant human IL-1β and TNFα were provided by Genentech (South San Francisco). The anti-Flag MAb M2, M2 cross-linked to sepharose beads (M2 beads), and purified Flag peptide were purchased from Eastman Kodak Company (New Haven). Rabbit anti-Myc polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz). Antiserum against IRAK and IL-1RI was raised in rabbits as described (Cao et al., 1996a; Croston et al., 1995). Polypeptides containing the N-terminal 152 amino acids of MyD88 with a polyhistidine tag was expressed in Escherichia coli BL21, purified under denaturing conditions with nickel-coated beads as previously described (Cao et al., 1996a), and used for generating antibodies in rabbits (Zymed, South San Francisco). Human embryonic kidney 293 cells and HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 μg/ml penicillin G, and 100 μg/ml streptomycin (Life Technologies).

Expression Vectors

Mammalian expression vectors encoding N-terminal Flag-tagged IL-1RAcP, IRAK, and ΔTRAF6 have been described elsewhere (Cao et al., 1996a, 1996b; Huang et al., 1997). The control expression plasmid pRK5, the NF-κB–dependent E-selectin-luciferase reporter gene plasmid pELAM-luc, and pRSV-β-gal also have been described (Schall et al., 1990; Schindler and Baichwal, 1994; Rothe et al., 1995). Expression vectors for N-terminal Flag-tagged IL-1RI, IL-1RIΔ45, and IL-1RAcPΔ37 were constructed by inserting polymerase chain reaction (PCR)–generated cDNA fragments lacking the coding sequence for the signal peptide into the mammalian expression vector pFlag-CMV-1 (Eastman Kodak Company, New Haven). The plasmid encoding N-IRAK (amino acids 1–215) was generated by inserting the PCR-amplified corresponding IRAK cDNA into pRK5. IRAK K239S was constructed using the Quick-Change site-directed mutagenesis kit (Stratagene, La Jolla). Expression vectors for epitope-tagged MyD88 (Genbank accession number U84408), MyD88-N (amino acids 1–151), or MyD88-C (amino acids 152–296) were constructed in-frame with a N-terminal Flag or Myc epitope or a C-terminal Flag epitope in pRK5.

293IL-1RI/AcP Cell Line

293IL-1RI cells (Cao et al., 1996a) were seeded in a 10 cm dish and transfected with 5 μg of expression plasmid for N-terminal Flag-tagged IL-1RAcP and 0.5 μg of plasmids containing a hygromycine-resistance gene controlled by the SV40 promoter and enhancer by calcium phosphate precipitation (Ausubel et al., 1994). The cells were cultured in DMEM with 100 μg/ml hygromycine B (Boehringer Mannheim) until cell colonies were visible by eye. The colonies were picked with pipette tips. The clones expressing the Flag-tagged IL-1RAcP were identified by flow cytometry with anti-Flag MAb and propagated like regular 293 cells.

Metabolic Cell Labeling

293IL-1RI/AcP cells were cultured in 10 cm dishes to 80% confluence. The cells were washed once with phosphate-buffered saline (PBS) and incubated in DMEM lacking methionine and cysteine with 5% dialyzed fetal bovine serum and 200 μCi/ml [^{35}S]methionine/cysteine protein labeling mix (New England Nuclear) for 16 hr. The cells were collected with PBS with 1 mM EDTA, pelleted by a 5 s spin in a microfuge, and resuspended in 37°C DMEM. The cell suspension was divided in two halves: one was treated with IL-1β (200 ng/ml) for 3 min and the other was left untreated. The cell lysate were aliquoted and immunoprecipitated with antibodies as described below. The immunoprecipitates were boiled in SDS sample buffer, separated on a 10% SDS gel, and exposed to x-ray film.

Purification and Sequencing of p35

293IL-1RI/AcP cells were cultured in suspension until a density of 500,000 cells/ml was reached. Cells from 40 liters of culture were pelleted by a 5 min 500 × g centrifugation and resuspended in prewarmed DMEM. Half of the cells were induced for 5 min with 200 ng/ml IL-1β, and the other half were not induced. The cells were lysed in lysis buffer (described below). The lysates were centrifuged at 2000 × g for 10 min, and the supernatants were centrifuged again for 2 hr at 100,000 × g in a Sorval Ti45 rotor. The supernatants were mixed with the M2 beads at 4°C overnight. The beads were collected in an empty column and washed with 20 column volumes of lysis buffer followed by 20 volumes of lysis buffer containing 1 M NaCl. The IL-1RAcP complex bound was eluted with three column volumes of lysis buffer with Flag peptide (200 μg/ml). The eluates were concentrated with Centricon 30 (Amicon) to 200 μl. The proteins were precipitated with 50 μl of ice-cold 100% (w/v) trichloroacetic acid with 4 mg/ml sodium deoxycholate, separated on a 4%–20% gradient SDS gel, and transferred to polyvinylidene difluoride (PVDF) membrane. The protein bands were visualized by Coomassie blue staining. The 35 kDa band was excised, alkylated with isopropylacetamide, and digested in 20 μl of 0.05 M ammonium bicarbonate containing 1% Zwitergent 3–16 (Calbiochem) (Lui et al., 1996) with 0.2 μg of trypsin (Frozen Promega Modified) at 37°C for 17 hr. The solution was then directly injected onto a 0.32 × 150 mm C18 capillary column and subjected to sequencing as described (Hou et al., 1994).

Reporter Assays

For reporter gene assays, 1×10^5 293 or HeLa cells were seeded into six-well (35 mm) plates. Cells were transfected the following

day by the calcium phosphate precipitation method with 0.5 μg pELAM-luc, 1 μg (for 293 cells) or 2 μg pRSV-β-gal (for HeLa cells), and the indicated amounts of expression constructs. After 24 hr (for 293 cells) or 46 hr (for HeLa cells), the cells were stimulated with IL-1β (20 ng/ml) or with TNFα (100 ng/ml) for 8 hr before harvest. Luciferase activity and β-galactosidase activity were determined with the Luciferase Assay System (Promega) and chemiluminescent reagents from Tropix, respectively.

Immunoprecipitations and Immunoblotting

For coprecipitation of transfected proteins, 3×10^6 293 cells were plated on 10 cm dishes and transfected the following day with the indicated amounts of expression constructs. The total amount of DNA was kept constant in each transfection by adding an empty vector. After 24 hr, cells were collected, washed once with PBS, and lysed for 20 min on ice in 1 ml of lysis buffer containing 50 mM HEPES (pH 7.9), 250 mM NaCl, 20 mM β-glycerophosphate, 1 mM sodium orthovanadate, 5 mM p-nitrophenylphosphate, 2 mM dithiothreitol, protease inhibitors (Complete, Boehringer Mannheim), and 1% NP-40. Cellular debris was removed by centrifugation twice at $10{,}000 \times g$ for 15 min. Half of the cell lysate (0.5 ml) was incubated with 1 μg of the specific antibodies and 20 μl of 50% (v/v) protein A-Sepharose slurry (Zymed, South San Francisco) or M2 beads (Eastman Kodak) for 2 hr at 4°C with gentle rocking, while the other half was incubated with an unrelated antibody and protein A-Sepharose as controls (data not shown). After extensive washing with lysis buffer, proteins bound to the beads were solubilized by boiling in SDS sample buffer, fractionated by SDS-PAGE, transferred to PVDF membranes, and blotted with the indicated antibodies. The reactive bands were visualized with horseradish peroxidase coupled to the appropriate secondary antibodies with an enhanced chemiluminescence Western blotting detection system (Amersham Life Science).

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